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Award Number: DAMD17-99-2-9027

TITLE: Drug Development and Conservation of Biodiversity in West
and Central Africa

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REPORT DATE: May 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2000		3. REPORT TYPE AND DATES COVERED Annual (1 May 99 -30 Apr 00)	
4. TITLE AND SUBTITLE Drug Development and Conservation of Biodiversity in West and Central Africa				5. FUNDING NUMBERS DAMD17-99-2-9027	
6. AUTHOR(S) Cyrus J. Bacchi					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Pace University New York, New York 10038-1502 E-MAIL: cbacchi@pace.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; Distribution Unlimited					12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) This research was conducted as part of Associated Project #3 of the International Cooperative Biodiversity Group (ICBG) program awarded to the Walter Reed Army Institute for Research (WRAIR). This project concerns the detection of growth inhibition of African trypanosomes and of pathogenic trichomonads by phyto-extracts from West Africa. During this period, 21 extracts received from WRAIR were screened against one <i>Trypanosoma brucei</i> strain and three <i>Trypanosoma rhodesiense</i> strains. Eight of these had IC ₅₀ values of ≤ 0.1 to < 20 $\mu\text{g/ml}$, and were of interest for further studies. Four extracts from previous studies were tested <i>in vivo</i> in a <i>T. brucei</i> mouse model, at up to 50 mg/kg/day i.p. x 3 days. None prolonged the life-span of infected animals. Additionally, based on trypanosome sterol requirements and evidence of plant sterols in active plant extracts, five anti-hypercholesteremic agents used in clinical medicine were tested in the trypanosome mouse model. None prolonged the life-span of infected animals at up to 100 mg/kg/day for 3 days. Sixteen extracts were tested vs. metronidazole-sensitive and -resistant isolates of <i>Trichomonas vaginalis</i> and a <i>Tritrichomonas foetus</i> isolate. Of these, five had MIC values of ≤ 0.1 mg/ml including an extract of <i>Dracaena mannii</i> which gave MIC values of 0.0125 to 0.006 mg/ml. These studies are continuing, with increased emphasis on <i>in vivo</i> testing of more highly purified plant extracts in an effort to determine the active agent(s) in these extracts.					
14. SUBJECT TERMS				15. NUMBER OF PAGES 13	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature Date

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(5) Introduction

Treatment of human and veterinary African trypanosomiasis and infection due to *Trichomonas spp* has remained stagnant for 50 and 40 years, respectively.

African sleeping sickness in humans and domestic animals is endemic in sub-Saharan Africa and the incidence of human cases is increasing dramatically. Treatment for human trichomoniasis (*Trichomonas vaginalis*) has remained static since 1955 (metronidazole), and resistance to this agent is increasing; there is no routinely available cure for bovine trichomoniasis (*Tritrichomonas foetus*) which causes miscarriage in cattle and is a significant economic problem. The purpose of this Associate Program (AP3) of the ICBG proposal was to screen extracts of known, medically active plants originating in West Africa (Nigeria and Cameroon) for activity against trypanosome and trichomonad isolates. With this in mind, extracts of native plants were prepared by the Phytochemistry group of the Walter Reed Army Institute of Research (WRAIR: AP2), for use in these screens. Accordingly, 21 extracts received from WRAIR were screened initially in *Trypanosoma brucei* and three isolates of *Trypanosoma rhodesiense*. Sixteen extracts were screened vs. two strains of *T. vaginalis* (metronidazole sensitive C1-NIH and resistant CDC-085) and one strain of *T. foetus*.

(6) Body

a) Screening of plant extracts for *in vitro* growth inhibitions vs. African trypanosomes. A total of 25 primary and secondary plant extracts were received from WRAIR. Of these sufficient quantities of 21 allowed testing against all four trypanosome isolates in a standard screen ([1]; Table 1). The data indicated that eight agents had significantly low IC₅₀ values (≤ 0.1 to ≤ 20 $\mu\text{g/ml}$) as to be of interest for continued study. The origins of these extracts were: *Cryptolepis sanguinolenta*, *Platex vellous*, *Fagara lemairei*, *Erythrina senegalensis*, *Glossocalyx brevipes*, *Dorstenia barteri*.

An additional 14 extracts were supplied by Dr. Johnson Ayafor of the University of Dschang, Cameroon, a collaborator in the ICBG program project (Table 2). All of these had IC₅₀ values of $\leq 20\mu\text{g/ml}$, and included methanol and water extracts of *Aframomum aulocacarpus* and *Glossocalyx brevipes*, as well as extracts of other local plants. Data for extracts AI+6, AI+7, ND1, ND2, and ND5 is incomplete, since the *T. b. rhodesiense* screens have not yet been run.

b) *In vivo* studies using mouse model infections. A total of 17 extracts were judged sufficiently active for screening *in vivo* (Table 3). Table 4 outlines the *in vivo* screening studies with plant extracts. Four extracts giving low IC₅₀ values in a previous study were run using the *T. b. brucei* Lab 110 isolate in a standard screen [1]. Agents were suspended in 2% methyl cellulose + 0.5% Tween 80, and administered i.p. for 3 days. SU-1461 was also given p.o. for 3 days. None of the extracts protected the mice and they died at the same time as infected, untreated controls. The remaining *in vivo* screens will be set up once additional supplies of extracts are received from WRAIR and Dr. Ayafor.

c) Sterol synthesis inhibitors: link to hemoflagellate parasites. Some phytosterols act as cholesterol analogs and can disrupt sterol uptake and synthesis in hemoflagellate parasites leading to death of the parasite. African trypanosomes depend on exogenous cholesterol [2]. Coppens et al. [3] showed the inhibitor Synvinolin (symvastatin) potentiates growth inhibition *in vitro* of *T. brucei* in the presence of drugs interfering with exogenous supply of cholesterol and that inhibition could be reversed by squalene, mevalonate, or cholesterol. Coppens and Courtoy [4] showed that *T. brucei* procyclic forms (insect vector) contain egosterol synthesized *de novo*, and also incorporate exogenous cholesterol into membranes. Culture-adapted parasites also grow more rapidly in the presence of LDL.

It is known that plant terpinoid saponins are part of the active agents found in plant extracts from Dr. Christopher Okunji's laboratory at WRAIR (AP2), and that such agents were found to have antileishmanial properties ([5]; Dr. J. Jackson, WRAIR, Pers. Commun.). The general inhibitory effects of terpinoid saponins on metabolism include: hypercholesteremic effects, disruption of blood lipid chemistry, hypoglycemic effect, inhibition of thymidine transport, and inhibition of Ca^{++} flux [5, 6]. In light of these widespread cytotoxic effects, we examined the activities of agents known to treat hypercholesteremia in humans on growth of *T. brucei* *in vitro* and *in vivo*. These agents are 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) inhibitors and have been found to be active in combination with the ergosterol antagonist ketoconazole against *Trypanosoma cruzi* [7]. We had examined the activities of HMGR antagonists vs. *T. brucei* *in vitro*, and found that Lamisil (IC_{50} = 1.3 $\mu\text{g/ml}$), Zocor (1.3 $\mu\text{g/ml}$), Mevacor (3.3 $\mu\text{g/ml}$), and Baycol (13 mg/ml) were potent growth inhibitors. We then tested these agents *in vivo* in a model *T. brucei* infection (Table 5). Since we were given commercially available pills or capsules containing these agents preparations, we ground the pills in a mortar and calculated the dose based on percent of active compound, dissolving or suspending the material in 2 % methylcellulose + 0.5 % Tween 80. The dose schedule was up to 100 mg/kg/day for 3 days (Table 5). None of the agents were active. Moreover, we tested ketoconazole at up to 60 mg/kg/day (i.p.) for 5 days and found no activity. During the following grant period, we intend to combine ketoconazole with the HMGR inhibitors to determine whether synergism is present, as shown previously for *T. cruzi* and *Leishmania spp* ([7]; J. Jackson, Pers. Commun.).

d) *In vitro* activity of plant extracts vs. *Trichomonas spp.* A total of 16 plant extracts were screened against *T. vaginalis*, metronidazole-sensitive (C1-NIH) and resistant (CDC-085) isolates, while 11 extracts were tested vs. *Trichomonas foetus* (KV1). All of these agents gave IC_{50} values < 1 mg/ml and several (SU-1460, 1461, 1464) were active at < 0.1 mg/ml . One very interesting finding was the very low IC_{50} obtained for SU-1460 vs. the metronidazole-resistant strain CDC-085. This value, 0.0015 mg/ml was 100-fold less than that found for the standard sensitive strain (C1-NIH). Of significance was the low IC_{50} values obtained for the *T. foetus* extracts (SU-1461, 1464). Further studies will examine secondary extracts of the active extracts in an attempt to improve activity and specificity. Additional studies will also involve *in vivo* testing through use of a subdermal trichomonas model infection in laboratory mice (N. Yarlett, pers. commun.).

(7) Key Research Accomplishments

- Identification of eight plant extracts having significant growth inhibitory activity ($\text{IC}_{50} \leq 20$ $\mu\text{g/ml}$) against African trypanosomes.
- Identification of five plant extracts having MIC values of ≤ 0.1 mg/ml toward *Trichomonas* and *Tritrichomonas* isolates.
- Several plant extracts effective *in vitro* vs. trichomonads were not active vs. trypanosomes, indicating a significant degree of specificity, e.g., extracts from *Mitracarpus scaber* and *Eupatorium adorum*.
- Determination that human hypercholesteremic agents, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) inhibitors, block *in vitro* growth of African trypanosomes at 1.4 - 13 $\mu\text{g/ml}$.
- The antihypercholesteremic agents, Lamisil, Mevacor, Pravachol, Zocor, and Lescol are not curative in mouse model infections, when used alone. Ketoconazole, which blocks synthesis of ergosterol in fungi is also not active *in vivo* in model infections.

(8) Reportable outcomes

U.S. Patent Application Serial No. 09/382,128

Filing Date: August 24, 1999

For: Antifungal and Antiparasitic Compounds

By: J.E. Jackson, M.M. Iwu, C.O. Okunji, C. Bacchi, J.D. Tally, Jr., J.F. Ayafor.

U.S. Patent Application Serial No. 09/428,203

Filing Date: October 27, 1999

For: Plant-derived Antiparasitic Antifungal Compounds and Methods of Extracting the Compound.

By: C.O. Okunji, J.E. Jackson, M.M. Iwu, C. Bacchi, J.D. Tally, Jr., J.F. Ayafor.

(9) Conclusions

Research over the past year has identified a number of plant extracts having significant *in vitro* growth inhibitory activity against African trypanosomes and pathogenic trichomonads. Although several of the extracts active against trypanosomes were tested in an *in vivo* screen with no activity, additional supplies of these extracts are needed to effect extended (5 or 7 day) dosing regimens. Moreover, additional purification of active extracts is needed to allow better control of *in vivo* dosing - at present high doses of these extracts are given, with incomplete solubilization of the extract.

In comparing *in vitro* activities of extracts, it is evident that a significant degree of specificity is present - for example, SU-1763 from *Mitracarpus scaber* is highly active vs. *T. vaginalis* isolates (MIC 0.1–0.9 mg/ml) but has little activity vs. African trypanosomes (IC₅₀: 195–300 µg/ml); the same relationship is also evident for *Eupatorium adorum*, SU-1752.

Exploration of antihypercholesteremic agents (HMGR inhibitors) as possible trypanocides seems promising, since some agents are highly effective *in vitro*. Additional dose regimens need to be tested *in vivo*, as do combinations of these inhibitors and ketoconazole. Pure preparations of HMGR inhibitors would facilitate administration in *in vivo* studies.

Economically, this is an important program for Nigeria and Cameroon: both are endemic for human and veterinary trypanosomiasis, while STD infections, including *T. vaginalis* and bovine trichomoniasis are a significant source of human suffering and an economic drain. There is an urgent need for new and inexpensive drugs for trypanosomiasis. Treatment of human trichomoniasis depends solely on metronidazole, and there is no agent currently available for bovine trichomoniasis [metronidazole kills the microbial flora of the rumen and cannot be given to cattle]. Development of anti-protozoal agents from local plants would be a major factor in the well-being of these populations and a boost to local and national economics.

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Table 1. Activity of plant extracts vs. growth of African trypanosomes *in vitro*. Bloodform trypanosomes were grown in 24 well culture dishes (1 ml/well) in HMI-18 medium (Hirumi & Hirumi 1989). One half of the culture volume was replaced daily with fresh medium plus drug. Each extract was dissolved in 100% DMSO and diluted with medium. Cells were counted daily with a coulter counter. Data are as IC₅₀ values in µg extract/ml culture. Four strains were used: *T. b. brucei* Lab 110 EATRO, and three *T. b. rhodesiense* clinical isolates from the Kenya Trypanosomiasis Research Institute (KETRI). All data from 48 hr cultures. Control cell counts averaged 5 x 10⁶ cells/ml at 48 h. (Data thru April 30, 2000).

	IC ₅₀ (µg/ml)			
	EATRO 110	KETRI 243	KETRI 269	KETRI 243 As10-3
SU-1749	44	19.5	18.5	37
SU-1750	19	76	37	31
SU-1751	20	20.5	73	20.5
SU-1752	202	190	225	200
SU-1753	-	-	-	-
SU-1754*	0.008	0.09	0.0074	0.019
SU-1755	-	-	-	-
SU-1756	75	18.5	13.5	6.6
SU-1757	1.5	21.5	13	22
SU-1758	2.2	2.0	2.05	1.55
SU-1759	20.5	170	130	140
SU-1760	7.2	9.1	15.5	14.75
SU-1761	18.9	20	22	20.5
SU-1762	98	105	71	26
SU-1763	195	32% @ 500 µg/ml	235	300
SU-1764	225	225	225	220
SU-1765	200	200	200	180
SU-1766	-	-	-	-
SU-1767	-	-	-	-
SU-1768	0.78	0.76	0.715	1.3
SU-1769	7.5	7.3	15.25	6.1
SU-1770	16.5	19.25	16.0	6.8
SU-1771	54	60	56	29.5
SU-1772	50	47	35.5	17.5
SU-1773	210	210	210	180

*diluted with 0.1 M Tris-saline pH 7.4

Table 2. IC₅₀ values for Ayafor. Compounds were tested vs. trypanosome isolates grown in bloodforms in HMI-18 medium containing 20% fetal bovine serum as in Table 1. Coulter counts were made daily and IC₅₀ values determined after 48 h. (Data thru April 30, 2000).

		IC ₅₀ (µg/ml)		
		Lab110 EATRO	KETRI	
			243	243 As 10-3
			269	
SU-1460 A	0.59	0.18	0.62	0.59
SU-1460 B	21.0	26.5	10.0	16.0
S-1464 A	0.7	0.088	0.29	0.195
S-1464 B	0.235	0.18	0.155	0.335
DC-1	7.2	0.7	1.1	17
DC-2	7.0	1.95	1.25	2.05
PH-1	5.9	14.5	20	48
PH-2	2.3	11	9.4	13
XBX-2	1.49	3.9	5.6	2.05
AL+6	1.4	-	-	-
AL+7	67	-	-	-
ND1	3.2	-	-	-
ND2	11.5	-	-	-
ND5	1.9	-	-	-
Pentamidine	0.00048	0.00186	0.00192	0.003
Melarsen oxide	0.00077	0.0025	0.0066	0.0072

Table 3: Most active plant extracts for *in vivo* testing in trypanosome screen.

WRAIR supplied:

SU-1754, 1757, 1758, 1760, 1761, 1768, 1769, 1770.

J. Ayafor supplied:

SU-1460A, 1460B, 1464A, 1464B, DC1, DC2, PH-1, Ph-2, XBX-2

Table 4: Summary of testing with ICBG compounds vs. *T. b. brucei* mouse model

Groups of 3 mice are infected with 2.5×10^5 trypanosomes and treatment is begun 24 h post-infection. Treatment is given once daily x 3 days, usually at 1, 5, 10, and 25 mg/kg, i.p.

All of the following were inactive at the dosages tested. They did not prolong life beyond that of the untreated controls:

- SU-1460A, SU-1460B
- AZ2 (SU-1460: up to 50 mg/kg x 3 days)
- SU-1461 (up to 50 mg/kg i.p. and p.o.)

Table 5: Summary of testing anti-hypercholesteremia agents vs. *T. b. brucei* mouse model

- Groups of 3 mice were infected with 2.5×10^5 trypanosomes and treatment was begun 24 h post-infection.
- Pills were ground using a mortar and pestle, and the compounds were suspended in 2% methylcellulose containing 0.5% Tween 80.
- Agents were administered orally once daily for 3 days.
- Doses used were 25, 50, 100 mg/kg/day for Lamisil, Mevacor, Pravachol, Zocor, and Lescol.
- Lopoid was used at 200, 400, 600 mg/kg/day.
- Doses were calculated based on the percent active compound in each pill or capsule.
- None of the agents increased survival time beyond that of the infected untreated controls.
- Ketoconazole was also tested in this system at 15, 30, 45, and 60 mg/kg/day x 5 days, p.o. and i.p. dose regimens were used. None were effective.

Table 6. Inhibition of *Trichomonas* growth by new primary plant extracts. The assay system used was the standard MIC (minimal inhibitory concentration) assay (Meingassner et al 1978) for *Trichomonas* in which serial dilutions were prepared in medium using sterile 96 well plates. Twelve dilutions were made, with a concentration range of 2.5 to 0.0012 mg/ml. Each well contained 10^4 organisms. Plates were incubated aerobically for 48h then examined. The MIC is defined as the minimum concentration of drug in which no motile organisms are visible after 48 h incubation. C1-NIH is metronidazole sensitive, CDC-085 is metronidazole-resistant and KV1 is the cattle parasite, *Tritrichomonas foetus*. Data expressed as MIC in mg/ml. ND, not determined.

Extract	Origin	MIC		
		C1-NIH	CDC-085	KV1
SU 1458	<i>Araliopsis tabouensis</i> AZ ₂	0.2	0.4	0.4
SU 1460	<i>Afromonum aulocacarpus</i>	0.1	0.0015	0.1
SU 1461	<i>Dracaena mannii</i> Mannispirostan A	0.0125	0.006	0.05
SU 1462	<i>Napoleonaea imperialis</i> MEOH	0.1	-	0.4
SU 1463	<i>Pachypodanthium staudtii</i> CH ₂ Cl ₂	0.80	ND	>0.80
SU 1464	<i>Glossocalyx brevipes</i> CH ₂ Cl ₂	0.0125	0.0125	0.0125
SU 1465	<i>Enantia chlorantha</i> MeOH	0.80	0.10	>0.80
SU 1466	<i>Eupatorium odoratum</i> MEOH	0.4	0.4	0.4
SU 1467	<i>Cleistopholis patens</i> EtOH	>0.80	0.10	>0.80
SU 1468	<i>Leidobotrys staudii</i> CH ₂ Cl ₂	0.40	ND	>0.80
SU 1469	<i>Ancistrocladus bateri</i> ABSBM	0.40	ND	0.40
SU 1751	<i>Eupatorium odoratum</i>	0.3	0.6	-
SU 1752	<i>Eupatorium odoratum</i> MEOH	0.3	0.3	-
SU 1758	<i>Fagara lemairei</i>	0.6	-	-
SU 1759	<i>Fagara lemairei</i> MEOH	0.6	-	-
Su 1763	<i>Mitracarpus scaber</i> (Pet. Ether)	0.1	0.9	-
Metronidazole		0.003	0.40	0.003